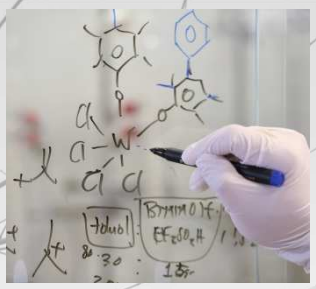
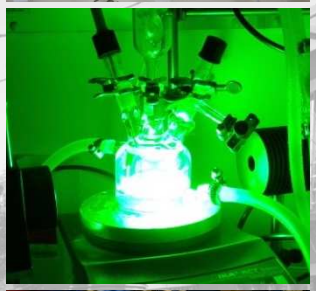
Friedrich-Alexander-Universität
Technische Fakultät

Design of a future enzyme process

CBI project course spring 2022

Partner: ROCHE Diagnostics GmbH, Penzberg





The project course is a curriculum event of no commercial interest organized by the *Department Chemie- und Bioingenieurwesen* (CBI) of *Friedrich-Alexander-Universität Erlangen-Nürnberg* (FAU) in cooperation with an industrial partner. The industrial partner of the CBI project course spring 2022 is ROCHE Diagnostics GmbH, located in Penzberg, Germany. The aim of this project course was the design of a **multipurpose bioreactor** for the production of **different enzymes via fermentation**. Currently industry obtains these enzymes from different plant-based sources.

	Source	Demand [MU]
Ascorbate Oxidase	Curcubita species	8400
Urease	Jack bean	2640
Peroxidase	Horseradish	250

For the design of the reactor, both multipurpose steel bioreactors with maximum 10 m^3 volume and single use bioreactors (SUB) with maximum 2 m^3 volume, were considered. Depending on the calculated times needed for the single process steps and the temporal linkage of the steps a **total production time for the three enzymes of 186 days** was determined.

Aim of **media and expression** was the evaluation and selection of possible expression systems for the biosynthesis of the peroxidase from *Armoracia rusticana* (POD), urease from *Canavalia ensiformis* and ascorbate oxidase from *Cucurbita sp.* (ASO) in a large-scale industrial environment. Subsequently high cell density media for the chosen expression systems were evaluated and selected. For **production of POD** the eucaryotic expression organism *Komagatella pfaffii* (also known as *Pichia pastoris*) was chosen, making posttranslational modifications and secretion into the medium possible. Futhermore, the selected strain CBS7435 MutS FWK3 facilitates a more homogenous glycosylation. The gene of interest will be integrated into the genome which is realised by homologous recombination of a linearized vector carrying the target gene PRXC1A. To select the transformants with the integrated gene, a selection marker like geneticin will be used. After gene integration, no further selection pressure is needed during the fermentation process to maintain the gene stability. For the secretion the acid phosphatase pre sequence (prePho1) will be N-terminally fused. The C-Terminus will be extended by an His_6-tag for the purification by chromatography. Additionally, the transcription factor Hac1 for the expression of auxiliary proteins can increase the production level due to an optimized folding process. Because of the need for co-expression of accessory proteins in the same compartment as urease and the successful expression of a similar ascorbate oxidase, *Escherichia coli* was chosen as expression organism for **production of urease and ASO**. For recombinant expression of urease (gene of interest: *JBURE-I*) and ASO (gene of interest: *AAO)* in *E. coli* a plasmid-vector is required, including a lactose induced T7 promoter and a tryptophan auxotrophy selection marker. For simplified chromatographical purification and improved solubility a His_6-SUMO-tag is added to the ASO whereas urease does not need any tags. Urease however requires a second plasmid for the expression of the vital accessory proteins UreD, UreF and UreG (taken from

Arabidopsis thaliana with 74 % sequence identity of its urease compared with the one from *Armoracia rusticana*). This second plasmid includes a T7 promoter, all three GOI and an uracil auxotrophy selection marker.

To **promote the vector design**, a high cell density defined salt medium was chosen for both microorganisms. Consequletly *Komagatella pfaffii* grows in a basal salt medium with glycerol whereas *E. coli* grows in Riesenberg medium with glucose basis. For successful expression of active POD in *Komagatella pfaffii* 10 µM of the prosthetic group hemin has to be supplemented, since the organism is not capable of providing it itself. For the production of active urease, 40 mg L^{-1} $NiCl_2$ have to be added to the Riesenberg medium. The expression of ASO in Riesenberg medium requires no further additives since the necessary copper is already contained in sufficient quantities in the medium (120 mg L^{-1} $CuCl_2$). The supplements are important for the structure and the active centre of the enzymes. The media components are sterilized using a **continuous sterilization process** via steam injection. The media are held at a temperature of 121 °C and a pressure of 2 bar for 15 minutes.

For the design of a **multipurpose fermentation plant** capable of producing different recombinant proteins in bacteria at different times the losses due to other operations further down the process such as purification had to be considered.

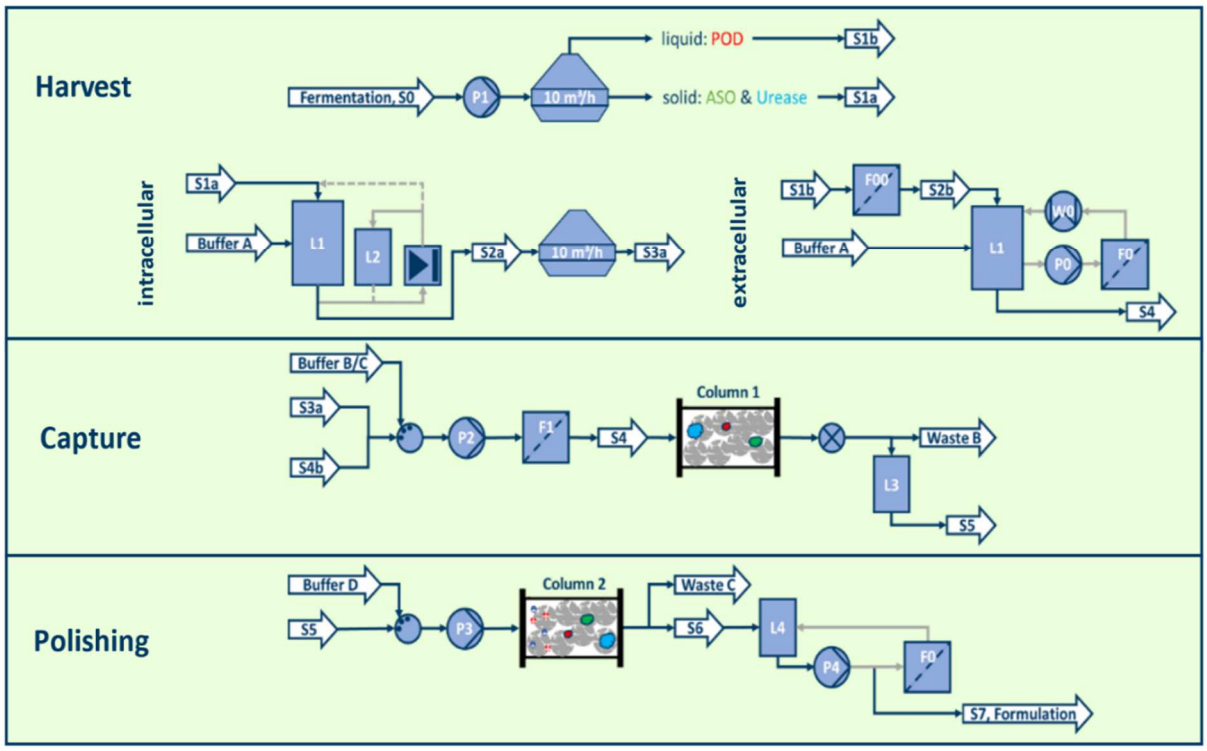
	Demand / kg	Total biomass / kg	Batches / -	Time per batch / h
Ascorbate Oxidase	165.4	16500	276	28.6
Urease	108.2	3400	57	28.6
Peroxidase	1.2	420	3	38.3

Taking k_La and oxygen transfer rate (OTR) values into account[1, 2], the **maximum concentration of biomass** per litre was calculated. Due to the resulting OTR value of 8 mmol L^{-1} h^{-1} for the SUB reactors, a 7.5 times lower biomass concentration was assumed, hence the **option of SUB reactors** was considered to be inefficient. In order to not exceed a reactor volume of 10 m^3 for the **steel bioreactor**, this volume was set to be the volume of the last steel reactor, resulting in reactor sizes of 80 L, 400 L, 2000 L and 10000 L. With the said maximum volume and the concentration of biomass per litre, the **number of batches** could be calculated. Furthermore, the time per batch could be calculated via growth kinetics formulas. The CIP/SIP process was assessed and a time of 2.4 h per reactor was obtained.

The **purification** of multiple recombinantly sourced enzymes had to meet requirements made by diagnostic. The suggested process divides into **three main operations**. Firstly, the harvest processes 10 m^3 of fermentation broth with intracellularly and extracellularly occurring enzymes to about 450 L of conditioned enzyme suspension via mechanical and thermal unit operations including **centrifugation, mixing, homogenization, filtration and concentration**.

[1] De Wilde D., Dreher T., et al.: Superior Scalability of Single-Use Bioreactors. BioProcess International (2014) No. 5, pp. 14-19.
[2] Crueger W., Crueger A.: Crueger's Biotechnology: A textbook of Industrial Microbiology. Sinauer Associates Inc 1990, p. 82.

Here, value is placed upon reducing the volume, fast cooling to under 12 °C and transferring all enzymes into sodium phosphate buffer to minimize loss of active enzyme. Secondly, the capture separates the enzyme from nearly all other “organic” components, e.g. other proteins, DNA or RNA, via **immobilized metal ion affinity chromatography**. Thirdly, the enzyme suspension is polished for formulation by separating it from step two’s eluent and possibly leached metal ions via **size exclusion chromatography** as well as concentrating it to target concentration via **tangential flow filtration**. The standardised and semi-continuous process achieves estimated yields of ASO, 60 % in 8 h, urease 54 % in 10.7 h and POD 72 % in 5.2 h therefore it could serve up to two fermenter lines while further improvements, as described in our report, are possible.



The solid **formulation** was chosen to provide the longest possible shelf life of the product and to minimize the amount of excipients needed. **Freeze-drying** was chosen as the drying method, because, compared to other drying methods, high temperatures and shear stresses cannot affect and damage the enzymes. To protect the enzymes, two **excipients** are added to the solution before freeze-drying. First, 1 wt.-% PEG 8000 is added to prevent freeze damage to the enzymes and second, 5-8 wt.-% trehalose is added to protect the enzyme activity. For optimal drying, the solids content of the solution should be 10 % after addition of the excipients. The temperature and pressure sequences of the protocol are based primarily on the vapor pressure of the solvent and the glass transition temperature of the solution. A total of 30 kg of solvent is sublimed per batch. For the **entire process of the formulation 34 h are estimated**. Since a new batch arrives every 12 h, a total of 3 freeze dryers are required.

Within one year 1.8 million vials can be produced with an activity of 21 kU/vial for ASO, 2 kU/vial for urease and 10 kU/vial for POD.

The product enzymes ascorbate oxidase and urease are produced intracellularly by *E. coli*, therefore **cell diruption** must happen before purification. The **high-pressure homogeniser** (HPH) was chosen as the most suitable method for the cell lysis, since every other method showed bottlenecks in terms of process applicability. Based on an empirical formula, the cell lysis quality was then calculated for a cell density of 150 g_{cells}/L_{buffer} and a pressure of 1200 bar.[3] The **release of enzymes is 99 %** with two passes. During one pass in HPH, the temperature rises by approx. 30 °C (2.4 K per 100 bar[4]). To avoid denaturation occurring above 70 °C[5], cooling between the two passes is mandatory. For the determination of the **enzyme activity** in the process, three different samples are required. The first one is collected directly after the fermenter to verify the success of the fermentation. For this sample, the purification needs to be conducted in small scale. The second sample is necessary to add the right amounts of excipients before the formulation. The third sample taken from the lyophilisate ensures the quality of the final product. For all three enzymes, established **photometric or colorimetric methods** for the determination of the enzyme activity could be found. The enzyme assays are based on the conversion of a photoactive substance. The third task was the analytics during and after the fermentation. For the fermenter, **suitable sensors** for pH, temperature, pressure, optical density, dissolved O_2, level and foam were chosen. In case of peroxidase, a sensor to monitor the concentration of methanol is necessary. Furthermore, pressure and flow sensors for the pipes and level, pressure and temperature sensors for the storage and buffer tanks were selected. An optical density sensor is additionally required in the two buffer tanks before and after the HPH. To monitor the washing step of the affinity chromatography, an UV-absorption sensor was chosen.

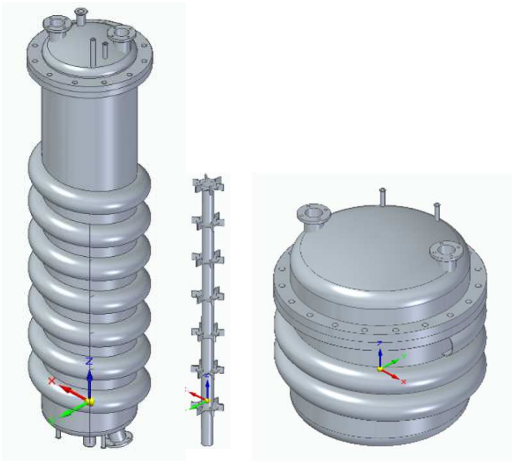
To gain information about the impact of the designed production plant, a **life cycle assessment** (LCA) was carried out. The functional unit of the LCA was the **amount of produced enzymes per year in kilogram**. Ten years were set as the lifetime of the plant equipment. The LCA was carried out with the software *openLCA*, while using the *ecoinvent* database as the main data source. System boundaries were set to **cradle-to-grave** for the plant and **cradle-to-gate** for the enzymes. Streams that account for less than 1 % of the mass, energy or emissions of a unit process are cut off. Allocation between the different enzymes is based on the number of batches produced in the corresponding process or the amount of throughput. The biggest factors regarding the **CO_2-emissions** of the production process are the manufacturing of substrates, the reactor cleaning and the fermentation steps. Most of the

[3] A. Middelberg, Microbial Cell Disruptionby High-Pressure Homogenization, 2000
[4] S. Jahnke, Produktentwicklung mit Homogenisatoren, GEA Niro Soavi GmbH, 2008
[5] CustomBiotech Catalog 16th Edition Clinical Chemistry and Immunology, Roche Diagnostics GmbH, https://custombiotech.roche.com/content/dam/acadia/productcatalog/986/00/CB_catalog_CC_IM%20ACaDia%20final.pdf

emissions originate from the energy used for the supply of glucose, the formulation of caustic soda for cleaning and the microorganisms' metabolism. A reduction in CO_2-emissions can be achieved by using renewable energy sources in the production process and the supply chain. Alternatively, other cleaning agents and different microorganisms that metabolize a different substrate can be utilized to decrease the GWP of the enzyme production. The LCA should be accompanied by a more detailed study, including scenarios with alternative substances and screening of literature for more reliable background data.

Common calculations and DIN standards were considered in order to optimally design the pipes and heat exchangers. For **heat transfer**, half-pipe coils were selected for all fermentation reactors and purification storage tanks. Tube bundle heat exchangers were used for heating and cooling of the fluid flows. The total heating requirement corresponds to an estimated value of 3470 kWh and the cooling requirement to 6560 kWh per batch. Stainless Steel 1.4404 (316L) with a maximum carbon content of 0.02 % was chosen to satisfy the requirement of **carbon-free piping material**. The pipe diameters were selected based on the PN6 pressure rating, between DN10 and DN40 depending on the volume flow. The estimated pipe length required for the whole process is around 210 m. Taking into account the fluid viscosity, volume flow, delivery head, pressure loss, corrosion resistance and CIP/SIP capability, a **diaphragm metering pump or a centrifugal pump** was chosen depending on the application. Diaphragm valves were selected for the whole system.

Four different bioreactors were designed for fermentation and five different vessels for purification. The requirements for the respective apparatuses were implemented using the relevant DIN standards and the AD 2000 regulations. For all **apparatuses**, a **two-part design** with flange was chosen in order to be able to reach the internals of the reactors and vessels for maintenance work. The basis for the drawing of the apparatus is a cylinder to which a bottom and a lid in the form of a Klöpper head are attached. The necessary connections for the material flows and the sensors are made in the lid and the bottom.



The stirrer in form of a Rushton turbine is installed in both apparatuses in combination with baffles. The bioreactor has a much higher height to diameter ratio than the tank, as this is necessary for aeration via ring sparger. Cooling coils in the form of a half-pipe coil jacket are attached to the outside. The apparatuses are made of stainless steel.

An **automation concept** of a multi-purpose production line for three different enzymes was established. During the process, the desired enzymes must be produced in required quantity and quality. To achieve this in a safe and smooth way, it was of great importance to gain an in-depth understanding of the individual sub-processes. Based on the required specifications, **suitable sensors** for each sub-process have been selected. These were linked to the actuators in such a way that the desired operating points of the fermentation, purification and other downstream processes were maintained. Once the sensor-actuator connections had been selected, various control concepts were considered. Examples for **controlled parameters** in the fermenters are pH, temperature, or dissolved oxygen. In addition to that, it was necessary to ensure a reasonable interconnection of the respective sub-processes. For this purpose, further components (e.g., storage containers) had to be included to ensure that an intermediate product can be prepared for the next step. Besides conventional automation of the production line, various aspects of **industry 4.0** were presented and it was clarified whether these can also be implemented in a biotechnological process.

In order to obtain information about the **equipment costs**, a wide range of manufacturers were contacted, who mostly were able to provide some information about purchase prices of different components. This resulted in a total **of 17.2 mil. €**. Based on this data, two different widely known and used **estimation methods** were used: LANG and HAND-model. Both methods are so called structure methods and provide estimation accuracies in the range of ±30-40 %.[6] The methods use one or more factors which are multiplied by the known equipment costs. Due to these factors, it is possible to take different cost drivers, for example instrumentation, automatization as well as piping, into account. For the LANG-model a **total project investment cost of 97.7 mil. €** was calculated, while the HAND-model results in a **total capital investment cost of 75.4 mil. €**. In addition to the investment costs, the **operating costs** also play a major role when it comes to assessing whether the construction and operation of a plant is worthwhile. Operating costs include staff costs, energy costs and material costs. Total operating costs were calculated at **33.6 mil. €** / **year**, with material costs accounting for the largest share (88 %). The reason for this large share is the huge amount of medium required to carry out the high number of fermentation batches to produce the enzymes. Based on sales prices, an amortisation period of **less than one year** was calculated. Since the costs could not be calculated in full due to the lack of information about some component costs, this result should definitely be viewed critically. With the selling price for ascorbate oxidase being the main reason for such a short payback period, a minimal selling price for this enzyme was approximated based on targeted amortisation periods of five and ten years.

[6] David Oliver Kunysz (2020), Kostenabschätzung im chemischen Anlagenbau, Springer Verlag

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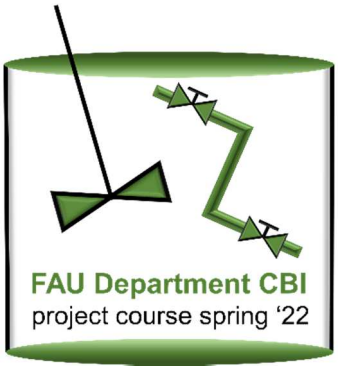

Design of a future enzyme process – Partner project with ROCHE Diagnostics

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